REMARKS

I. Status of the Claims

Prior to the present Action, claims 1-32 and 43-50 were pending (Fourth Action at Summary page). The Action at page 2 is therefore in error in listing claims 1-32 and 43-49 as pending. According to a species election requirement, claims 10-15, 20-23 and 44 are said to be withdrawn as reading on the non-elected species, although these claims have been examined (see **Section III** for discussion of species). Claim 32 is said to be free of the art (Fourth Action at page 2).

Presently, no claims have been canceled or amended. Claims 51-57 have been added, which are fully supported by the application as filed and unified with the examined claims. Claims 1-32 and 43-57 are therefore in the case. According to the revisions to 37 C.F.R. § 1.121(c), a copy of the pending claims is provided in the amendment section.

II. Support for the Claims

Support for the new claims exists in the pending claims and throughout the original application as filed. Any fees necessary for the introduction of the new claims should be deducted from Williams, Morgan & Amerson, P.C. Deposit Account No. 50-0786/3999.002383.

Claim 51 is a new independent claim directed to the subject matter of dependent claim 24, *i.e.*, to therapeutic kits of a first aminophospholipid targeting agent-therapeutic agent construct in combination with a second anti-cancer agent other than the first aminophospholipid targeting agent-therapeutic agent construct.

Independent claim 52 is a new therapeutic kit claim directed to the subject matter of dependent claim 2, *i.e.*, to kits in which the aminophospholipid targeting agent-therapeutic agent construct binds to phosphatidylethanolamine. The functional definition of the

aminophospholipid targeting agent-therapeutic agent construct as binding to the phosphatidylethanolamine aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor is supported by claim 49 and throughout the specification.

New claim 53 combines claim 49 and claim 24, and further defines certain preferred examples of the second anti-cancer agents in the therapeutic kits. The functional definition of the aminophospholipid targeting agent-therapeutic agent construct as binding to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor is supported by claim 49 and throughout the specification. The definition of a second anti-cancer agent other than the aminophospholipid targeting agent-therapeutic agent construct is first defined as in claim 24. Two groups of preferred second anti-cancer agents are then recited: (i) an agent that increases aminophospholipid expression, or injures or induces apoptosis in the tumor blood vessel endothelium, as supported by the specification at least at page 40, lines 4-8; and (ii) an agent that kills tumor cells or anti-angiogenic agent that inhibits metastasis of tumor cells, as supported by the specification at least from page 39, line 26 to page 40, line 1.

Dependent claim 54 separately recites a second anti-cancer agent as in claim 53 (b)(i), which increases aminophospholipid expression, or injures or induces apoptosis in the tumor blood vessel endothelium, supported by the specification at least at page 40, lines 4-8.

Claim 55 exemplifies agents in accordance with claim 54 as taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium ionophore or as a calcium-flux inducing agent (specification at page 40, lines 4-11), H_2O_2 or thrombin (Example XIV), an inflammatory cytokine (page 48, line 1; page 154, lines 16-23; Example XIV) or interleukin-4 (page 48, line 1; page 154, line 18).

Dependent claim 56 separately recites a second anti-cancer agent as in claim 53 (b)(ii), which kills tumor cells or is an anti-angiogenic agent that inhibits metastasis of tumor cells, as supported by the specification at least from page 39, line 26 to page 40, line 1.

Claim 57 exemplifies agents in accordance with claim 56 as an anti-tumor cell immunoconjugate, a chemotherapeutic agent or an anti-angiogenic agent, which are supported throughout the specification, with particular written description support at least from page 40, line 13 to page 42, line 21.

It will therefore be understood that no new matter is included within the new claims.

III. Restriction and Species Issues

The Action states that claims 10-15, 20-23 and 44 "are withdrawn from further consideration, as being drawn to a nonelected species. Applicant timely traversed the restriction (election) requirement in Paper No. 15. This application contains claimed [*sic*] 10-15, 20-23 and 44 drawn to an invention nonelected with traverse in Paper No. 15" (Fourth Action at page 2, emphases added). Each of these aspects of the Action is in error.

First, claims drawn to non-elected species cannot be withdrawn from "further" consideration, but remain pending and are rejoined upon allowance of a generic or other linking claim. 37 C.F.R. § 1.141(a). Moreover, many of the claims drawn to the originally non-elected species have already received an examination on the merits. For example, claim 14, which is currently said to be withdrawn from consideration, is rejected under 35 U.S.C. § 102(e). Second, the species elections were made without traverse in the response to the Second Restriction Requirement (see Applicants' response dated August 03, 2000, Section V, last paragraph, first sentence).

IV. Rejection of Claims 1-9 and 14 Under 35 U.S.C. § 102(e)

Claims 1-9 and 14[1] stand rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent No. 6,300,308 to Schroit ("Schroit"). Although Applicants respectfully traverse, the Action's concerns are overcome.

The following response should not be interpreted as an acquiescence that the effective filing of date Schroit is earlier than the effective filing date of the present application, nor as waiving any rights to establish a date of invention earlier than the effective filing date of Schroit.

Within the claims agreed to have been examined, *i.e.*, claims 1-9, 14, 16-19, 24-32, 43 and 45-50, each of claims 16-19, 24-32, 43 and 45-50 are free from this ground of rejection. Without acquiescing with the present rejection in any way, Applicants presently introduce claims 51-57, which are based upon and/or extend claims 24 and 49. Claim 51 places claim 24 in independent form, and claims 52-57 further define or extend claim 49. As detailed below, claims 1-9 and 14, and new claims 51-57, are novel over Schroit.

Claim 1 recites a kit comprising a first anti-cancer agent in the form of a targeting agent-therapeutic agent construct that comprises a targeting agent that binds to an aminophospholipid operatively attached to a therapeutic agent; and either a targeting agent-detectable agent construct that also binds to an aminophospholipid or a second anti-cancer agent other than the first targeting agent-therapeutic agent construct. Examination has focused on kits with second anti-cancer agents.

Schroit does not teach or suggest a kit comprising a first anti-cancer agent in the form of a first targeting agent-therapeutic agent construct that comprises a targeting agent that binds to an aminophospholipid operatively attached to a therapeutic agent, in combination with either a

[1]The rejection of claim 14 shows that each of claims 10-15, 20-23 and 44 cannot be withdrawn from consideration.

targeting agent-detectable agent construct that also binds to an aminophospholipid or a second anti-cancer agent other than the first targeting agent-therapeutic agent construct.

The rejection is largely based on the position that the first and second anti-cancer agents recited in present claim 1 can be exactly the same agent and, consequently, that the kits of claim 1 would be anticipated by a kit containing two aliquots of the same aminophospholipid targeting agent-therapeutic agent construct. Such a position is in error.

Despite the plain meaning of the claim, which recites that the second anti-cancer agent is an anti-cancer agent other than the first targeting agent-therapeutic agent construct, the Action takes the position that the claim "is not limited to two distinct constructs as the second anticancer agent encompasses any anticancer agent" (Fourth Action at page 3). According to the broadest reasonable interpretation of the claims, the second anti-cancer agent encompasses any second anti-cancer agent other than a targeting agent-therapeutic agent construct that binds to an aminophospholipid. To determine otherwise would be inconsistent with the language of the claim itself and the controlling case law, which requires that the broadest reasonable interpretation of the claims must be consistent with the interpretation that those skilled in the art would reach. *In re Cortright,* 49 USPQ2d 1464, 1468 (Fed. Cir. 1999).

The Action's claim interpretation, which ignores the claim term "other than", attempts to read a limitation out of the claims, which is prohibited. Every element of a claim has meaning; the language of the claim as a whole must be considered and an interpretation should not be reached that renders a clause superfluous. *Genentech Inc. v. Chiron Corp.*, 42 USPQ2d 1608, 1612 (Fed. Cir. 1997). Claims should not be read so as to improperly broaden the scope of the claims 'reading out' the limitations in claim language. *Lockheed Martin Corp. vs. Space Systems/Loral Inc.* 58 USPQ2d 1671, 1678 (Fed. Cir. 2001).

There is no reasonable interpretation of claim 1 to support the Action's contention that this claim is not limited to two distinct therapeutic constructs. The § 102(e) rejection, essentially based on the position that the first and second anti-cancer agents recited in claim 1 can be the same, is therefore in error and should be withdrawn. Should the Office maintain its interpretation of the claims and the resultant § 102(e) rejection, Applicants respectfully request that the Office identify the particular statutory or judicial authority and scientific reasoning underlying the proposed claim interpretation.

Aside from the improper claim interpretation, which underlies most of the rejection, the Action's additional comments also fail to support the § 102 rejection. A rejection on the grounds of anticipation requires the disclosure, in a single reference, of every element of a claimed invention and requires that each and every facet of the claimed invention be identified in the applied reference. *Minnesota Mining & Mfg. v. Johnson & Johnson Orthopaedics, Inc.*, 24 USPQ2d 1321 (Fed. Cir. 1992). The Action's reference to different biological components mentioned in isolation in different sections of Schroit is insufficient to support an anticipation rejection of the present claims, which are drawn to "a kit", *i.e.*, a combination of components that are designed for use together:

> "The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained."
>
> Specification at page 138, lines 19-22.

The lines and phrases in Schroit quoted at pages 3 and 4 of the Action do not teach or suggest kits with the elements of the present claims.

Schroit from column 7, line 67 to column 8, line 1 concerns one container with a PS composition and another container including "a matrix, solution, or other suitable delivery

device" for applying "the composition" to the body. That is, one PS composition and one delivery device. The Action states that Schroit refers to PS compositions, and thus contends that Schroit discloses kits that can contain "a second anti-aminophospholipid antibody conjugate" (Fourth Action at page 3). Even if Schroit refers to "PS compositions", this would not anticipate the claimed invention.

Notably, the Action has still not identified any teaching in Schroit concerning a first aminophospholipid targeting agent-therapeutic agent construct. The "PS compositions" referred to at column 7 are either PS-polypeptide conjugates or anti-PS antibodies alone. Furthermore, as Schroit concerns only PS, *even if* there was a suggestion of a first aminophospholipid targeting agent-therapeutic agent construct, and *even if* the "PS compositions" referred to two such constructs, *these would still be the same*. There is no teaching or suggestion in Schroit of a kit comprising a first anti-cancer agent in the form of an aminophospholipid targeting agent-therapeutic agent construct and a second anti-cancer agent other than the first targeting agent-therapeutic agent construct, as in the claimed invention.

The Action's further comments regarding claim 21, PS-polypeptide conjugates, separate moieties to be conjugated and diphtheria toxoid (Fourth Action at pages 3 and 4) have largely been taken out of context of the Schroit document (see below), but in any event, these separate aspects of Schroit do not teach or suggest the kits of the claimed invention.

Schroit at claim 21 does not recite an "antibody-therapeutic construct" (Fourth Action at page 3). Claim 21 of Schroit concerns a method for generating a lipid-specific antibody response by administering a PS-polypeptide conjugate in which PS is conjugated to the carrier BSA, KLH, BGG, diphtheria toxin or β2-glycoprotein I. The administered composition is thus not an antibody-therapeutic agent construct.

Schroit's reference to an antibody that can exist in "separate moieties to be conjugated by user of the kit" (Fourth Action bridging pages 3 and 4) concerns immunodetection reagents alone, in particular, "antibody-label conjugates", which can be in fully conjugated form or as intermediates or separate moieties to be conjugated (Schroit at column 6, lines 50-51).

The discussion of diphtheria toxoid in Schroit is limited to its use as a carrier, *i.e.*, when conjugated to PS. There is no teaching regarding the use of diphtheria toxoid as any form of therapeutic agent alone.

Importantly, irrespective of the context of the PS-polypeptide conjugates, immunodetection moieties to be conjugated and diphtheria toxoid carriers in Schroit, the Action has still not identified any aspect of Schroit that teaches or suggests a kit comprising a targeting agent that binds to an aminophospholipid operatively attached to a therapeutic agent in combination with a second, distinct anti-cancer agent.

For at least the foregoing reasons, claims 1-9 and 14 are therefore novel over Schroit.

As claims 24 and 49 are free from the § 102(e) rejection, each of claims 51-57, which are based upon claims 24 and 49, are also novel over Schroit. Claim 51, which presents dependent claim 24 in independent form, is novel over Schroit for the same reasons as the Office determined for claim 24. Each of claims 52-57, which are directed to the therapeutic kits of claim 49 in conjunction with additional features of the targeting agent or second anti-cancer agent, are also novel over Schroit for the same and additional reasons.

Claim 52 is drawn to a therapeutic kit in which the targeting agent of the targeting agent-therapeutic agent construct binds to phosphatidylethanolamine expressed on the luminal surface of blood vessels of a vascularized tumor. Claim 52 is novel for the same reasons as claim 49, and further because Schroit does not teach or suggest a targeting agent-therapeutic agent construct in which the targeting agent binds to phosphatidylethanolamine expressed on the

luminal surface of blood vessels of a vascularized tumor, let alone teach or suggest such a construct as part of a kit in accordance with the present claims.

Claim 53 is directed to a therapeutic kit in accordance with claim 49 in which the second anti-cancer agent is defined according to certain preferred features, particularly as an anti-cancer agent that (i) increases aminophospholipid expression, or injures or induces apoptosis in the tumor blood vessel endothelium; or that (ii) kills tumor cells or is an anti-angiogenic agent that inhibits metastasis of tumor cells. Claims 54 and 56 separately recite the two groups of second anti-cancer agents, and claims 55 and 57 provide particular examples thereof. Claims 53-57 are therefore novel for the same reasons as claim 49, and further because Schroit does not teach or suggest anti-cancer agents that increase aminophospholipid expression, injure or induce apoptosis in tumor blood vessel endothelium, or that kill or inhibit the metastasis of tumor cells, let alone teach or suggest such anti-cancer agents as part of a kit in accordance with the present claims.

The § 102(e) rejection over Schroit is therefore overcome and should be withdrawn.

V. Rejection of Claims 1-9, 16-19, 24-32, 43 and 45-49 Under 35 U.S.C. § 103(a)

The Action newly rejects[2] claims 1-9, 16-19, 24-32, 43 and 45-49 under 35 U.S.C. § 103(a)[3] as allegedly being legally obvious over the foregoing Schroit patent in view of U.S. Patent No. 5,632,991 to Gimbrone ("Gimbrone") and U.S. Patent No. 6,197,278 to Blankenberg *et al.* ("Blankenberg"). Although Applicants respectfully traverse, the Action's concerns are overcome.

The rejection of claim 32 is *prima facie* improper, as claim 32 is said to be free of the art (Fourth Action at page 2; Third Action at page 10). As claim 32 is free of the art, claim 43 is

[2]This is a new ground of rejection as the previously cited Umeda reference (Umeda *et al.*, *J. Immunol.*, 143:2273-2279, 1989) has been withdrawn from the combination.
[3]Claim 14 is rejected under § 102(e), but not under § 103(a).

also free of the art, as this is an independent claim of the same scope as dependent claim 32. Furthermore, claims 45-48, which each depend on claim 43, must also be free of the art.

The Action first contests Applicants' last response on the basis that one cannot show non-obviousness by attacking references individually where the rejections are based upon a combination of references (Fourth Action at page 4). In fact, the scope and content of the prior art must be ascertained before the obviousness or nonobviousness of the claimed subject matter can be determined. *Graham v. John Deere Co.*, 148 USPQ 459, 467 (U.S.S.Ct. 1966). Moreover, although the Action refers to the combination of references after formulating the rejection, some suggestion to combine the disclosure of two or more references in an attempt to establish *prima facie* obviousness must be established before the P.T.O. may combine references. *In re Fine*, 5 USPQ2d 1596, 1598-99 (Fed. Cir. 1988). The Action's assessment of the references is anyway incorrect and does not actually support their combination.

After setting forth the rejection, the Action takes the position that the three cited references are viewed to be in the same field of endeavor and are considered combinable because "each reference is directed to specific receptor molecule on the surface of human vascular endothelial cells associated with vascularized tumor" (Fourth Action at page 5). This statement not only improperly characterizes the references, but is derived from the present application and not from the cited art, and therefore has no place in an obviousness enquiry. It is impermissible to use the claims as a frame and the prior-art references as a mosaic to piece together a facsimile of the claimed invention. *Uniroyal Inc. v. Rudkin-Wiley Corp.*, 5 USPQ2d 1434 (Fed. Cir. 1988).

Schroit concerns PS and reports that, as opposed to the situation in normal cells, PS may appear at the surface of tumor cells (Schroit throughout, *e.g.*, column 16, lines 27-33). Gimbrone concerns E-selectin expression on activated endothelium in certain diseases or infections,

particularly in inflammation, and in connection with the metastatic spread of tumor cells (Gimbrone throughout, *e.g.*, Abstract, column 4, line 57 to column 5, line 7). Blankenberg concerns radiolabeled annexin[4] to image apoptosis and cell necrosis, mainly to detect inappropriate apoptosis, such as in neurons and the immune system, and also to detect insufficient apoptosis in tumor cells or virally infected cells (Blankenberg throughout, *e.g.*, column 5, lines 16-36 and column 12, lines 32-37).

None of Schroit, Gimbrone or Blankenberg teaches or suggests expression of PS, another aminophospholipid, E-selectin or any other specific receptor molecule on the surface of human vascular endothelial cells associated with a vascularized tumor. The statement in the Action at page 5 is therefore in error and the first ground advanced to support the combination of references is without merit. By referring to information from the present application, rather than from any of the cited references, this statement also evidences the Action's improper use of hindsight in formulating the rejection. To imbue one of ordinary skill in the art with knowledge of the invention in suit, where no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher. *W.L. Gore Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303, 312-313 (Fed. Cir. 1983).

The Action at page 5 next takes the position that Schroit, Gimbrone and Blankenberg are combinable because they are "used for the same purpose". This statement is also in error and thus fails to support the proposed combination or the resultant § 103 rejection. The purpose of Schroit is to generate PS-specific antibodies, which may bind to tumor cells; the purpose of Gimbrone is treat diseases and infections associated with E-selection expression on activated

[4]The citation of Blankenberg concerning radiolabeled annexin, which is an aminophospholipid binding protein, shows that each of claims 10-15, 20-23 and 44 cannot be withdrawn from consideration.

endothelium, particularly inflammation; and the purpose of Blankenberg is to image apoptosis *in vivo*. These three references are therefore not directed to the same purpose and the references have been improperly combined.

Importantly, even if combined, Schroit, Gimbrone and Blankenberg do not teach or suggest the presently claimed invention, and particularly do not teach or suggest a kit comprising a first anti-cancer agent in the form of an aminophospholipid targeting agent-therapeutic agent construct and a second anti-cancer agent other than such a targeting agent-therapeutic agent construct.

Schroit does not teach or suggest an aminophospholipid targeting agent-therapeutic agent construct, and particularly fails to teach such a construct in combination with a second, distinct anti-cancer agent. The Action cites Schroit at column 7, line 67 to column 8, line 1 as concerning kits containing "PS compositions" and contends that Schroit suggests kits that can contain "a second anti-aminophospholipid antibody conjugate" (Fourth Action at page 4).

As set forth above, even if Schroit refers to "PS compositions", there is no suggestion of a kit in accordance with the claimed invention. Any PS compositions in Schroit are PS-polypeptide conjugates or anti-PS antibodies alone. Even if Schroit could be interpreted to suggest a first aminophospholipid targeting agent-therapeutic agent construct, which is contested, any two such constructs *would be the same*, and Schroit cannot therefore suggest the claimed aminophospholipid targeting agent-therapeutic agent construct and second, distinct anti-cancer agent.

The Action next takes the position that "the instant therapeutic constructs within the kits do not exclude such constructs [PS-polypeptide conjugates] as taught by Schroit" (Fourth Action bridging pages 4 and 5). Should the Action be referring to the aminophospholipid targeting agent-*therapeutic* agent *construct* of the instant kit claims, as it appears, the aminophospholipid

targeting agent-therapeutic agent constructs of the claims clearly exclude PS-polypeptide conjugates as in Schroit. Should the Action be implying that a PS-polypeptide conjugate as in Schroit could be a second anti-cancer agent within the scope of the claims, this is far removed from rendering the invention of claims 1-9, 16-19, 24-32, 43 and 45-49 legally obvious.

It is well established under the law that an invention must be viewed in its entirety in formulating an obviousness rejection. The test for obviousness is not whether individual differences between the claims and the prior art are obvious but, rather, whether the claimed invention as a whole is obvious. *In re Buehler*, 185 USPQ 781 (CCPA 1975). "The entirety of a claimed invention, including the combination viewed as a whole, the elements thereof, and the properties and purposes of the invention must be considered." *In re Wright*, 6 USPQ 2d 1959 (Fed. Cir. 1988).

The Action has still not identified any aspect of Schroit that teaches or suggests a kit comprising a targeting agent that binds to an aminophospholipid operatively attached to a therapeutic agent in combination with a second, distinct anti-cancer agent. The line of argument that a PS-polypeptide conjugate of Schroit would be a second anti-cancer agent within the scope of the claims pertains to only one element of the claimed kit, and lacks any suggestion of the claimed invention *as a whole*.

Schroit therefore fails to teach or suggest the claimed invention. Indeed, the acknowledged novelty of several claims, such as claims 24 and 49, and the lack of a § 103 rejection of any claim based upon Schroit *alone* is an admission that Schroit does *not suggest* the presently claimed invention. The Action is therefore relying on Gimbrone and Blankenberg to cure the deficiencies of Schroit. However, Gimbrone and Blankenberg have been improperly combined with Schroit, and even if properly combined, Schroit, Gimbrone and Blankenberg together fail to teach or suggest the kits of the present invention.

Neither Gimbrone nor Blankenberg teach or suggest an aminophospholipid targeting agent-therapeutic agent construct. Gimbrone is silent as to aminophospholipids (Third Action at page 8) and Blankenberg concerns only diagnostic constructs of annexin. There is no mention in Blankenberg of aminophospholipid targeted therapeutic agents (Third Action at page 9). Thus, the secondary references each fail to teach or suggest the first anti-cancer agent of the claimed kits.

Gimbrone and Blankenberg also fail to teach or suggest a kit with a first and second anti-cancer agent of any description. In fact, it appears that these references lack any teaching or suggestion of a kit with first and second therapeutic agents of any type. Gimbrone was earlier cited as concerning second E-selectin-based therapeutic agents at columns 12, 14 and 15 (Third Action at page 8), but these sections of Gimbrone are limited an anti-E-selectin antibody, fragment or conjugate when used alone. The Action admits that Blankenberg does not teach the use of a therapeutic agent (Third Action at page 9). The secondary references thus fail to teach or suggest kits with two distinct anti-cancer agents, as required by the present claims.

In failing to teach or suggest both the first and second anti-cancer agents required by the present claims, Gimbrone and Blankenberg are incapable of curing the admitted deficiencies of Schroit. For at least the foregoing reasons, claims 1-9, 16-19, 24-32, 43 and 45-49 are therefore novel and non-obvious over Schroit, Gimbrone and Blankenberg, even if properly combined. Certain of the pending claims are also patentable over the cited references for various additional reasons.

Claims 2 and 49, drawn to kits in which the targeting agent of the targeting agent-therapeutic agent construct binds to phosphatidylethanolamine, are further patentable because the cited references do not teach or suggest a targeting agent-therapeutic agent construct in which the targeting agent binds to phosphatidylethanolamine, let alone such a construct as part of a kit in

accordance with the present claims. Indeed, Schroit teaches away from these aspects of the invention by the objective to produce highly-specific anti-PS antibodies (Schroit at column 2, line 36) and by the description of the resultant antibodies as being able to recognize PS but not DPOE (dioleoyl phosphatidylethanolamine) in a bilayer membrane (Schroit at column 25, lines 24-26).

Claims 16-18 are directed to kits in which the therapeutic agent in the aminophospholipid targeting agent-therapeutic agent construct is a coagulant, Tissue Factor or a Tissue Factor derivative such as truncated Tissue Factor. None of Schroit, Gimbrone or Blankenberg teach or suggest an aminophospholipid targeting agent-coagulant construct, or a kit thereof. To the extent that the Office deems the anti-E-selectin conjugates of Gimbrone to be relevant, Gimbrone teaches away from these aspects of the invention by disclosing an anti-coagulant immunoconjugate (Gimbrone at Example 5).

Claim 49, which recites a kit in which the aminophospholipid targeting agent-therapeutic agent construct is functionally defined as binding to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor, is further patentable as the cited references do not teach or suggest the expression of aminophospholipids on the luminal surface of tumor blood vessels, let alone teach or suggest a kit comprising the recited construct in combination with a second anti-cancer agent.

The surprising discovery that aminophospholipids are accessible, stable markers of tumor vasculature (specification at page 4, lines 24-26), as recited in claim 49, also underlies many surprising features of claims 1, 24-32 and 53-57. This finding in itself was particularly surprising, as the tumor vascular endothelial cells are normal cells, taught in the prior art to preserve PS in the inner leaflet (see Schroit at column 16, lines 28-31; Blankenberg at column 5, lines 62-63). Prior to the present invention, PS expression at the cell surface was reported to be

associated only with malignant or pathological cells, such as certain tumor cells and diseased neurons, or to result from myocardial infarction, reperfusion injury and stroke (see Schroit at column 16, lines 31-33; Blankenberg at column 5, lines 16-29).

The finding that PS was a marker of the normal cells of the tumor vasculature provided a means for effective therapy, overcoming the problems associated with tumor cell targeting, such as tumor cell resistance, antigen escape and effective penetration into the tumor. Moreover, the discovery of these aminophospholipid targets allows for the delivery of therapeutic agents in intimate contact with the tumor vascular endothelial cell membrane, allowing rapid entry into the target cell or rapid association with effector cells or components of the coagulation cascade (specification at page 5, lines 1-4). Importantly, the translocation of aminophospholipids to the surface of tumor vascular endothelial cells was further discovered to occur, at least in a significant part, independently of cell damage and apoptopic or other cell-death mechanisms (specification at page 5, lines 4-8). This discovery of sufficiently stable expression on morphologically intact tumor-associated vascular endothelial cells, which is again in contrast to the prior art (as evidenced by Schroit and Blankenberg), was an important step in the development of effective therapies (specification at page 5, lines 10-16).

In addition to providing effective tumor vasculature targeted therapy, as opposed to the difficulties associated with tumor cell targeting, the present discovery of sufficiently stable aminophospholipid expression on normal tumor vasculature endothelial cells gave rise to the combined anti-cancer therapeutics described in the present application and recited in the pending claims. In particular, a first targeting agent-therapeutic agent anti-cancer construct that binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor and another agent selected for "simultaneously or sequentially administering to the animal a therapeutically effective amount of at least a second anti-cancer agent" (specification from

page 39, line 15 to page 40, line 11; see also Section J, from pages 151-173). Therefore, the present invention, unlike the cited prior art, provides for the intelligent selection of first and second anti-cancer agents in a kit for use together.

This is highlighted in claims 53-57, which are directed to therapeutic kits in which the second anti-cancer agent is selected for combined use according to the guidance provided in the specification. A second anti-cancer agent administered at a biologically effective time *prior* to the aminophospholipid targeting agent-therapeutic agent construct is taught to (i) increase aminophospholipid expression, or injure or induce apoptosis in the tumor blood vessel endothelium (specification at page 40, lines 4-11); whereas a second anti-cancer agent administered at a biologically effective time *subsequent* to the aminophospholipid targeting agent-therapeutic agent construct is taught to (ii) kill tumor cells or to be an anti-angiogenic agent that inhibits metastasis of tumor cells (specification from page 39, line 26 to page 40, line 2).

Schroit, Gimbrone and Blankenberg, even if properly combined, do not teach or suggest anti-cancer agents that increase aminophospholipid expression, injure or induce apoptosis in tumor blood vessel endothelium, or that kill or inhibit the metastasis of tumor cells, let alone teach or suggest such agents as the second anti-cancer agent in a kit comprising a first anti-cancer agent in the form of a targeting agent-therapeutic agent construct that binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor. The present application, in contrast, teaches the rationale for selecting such agents in combination along with detailed teaching concerning second anti-cancer agents within each category.

The manuscript enclosed as **Exhibit A** provides actual data to support the reasoning in the application, showing that various factors and tumor-associated conditions known to be present in the tumor microenvironment are able to cause PS translocation in cultured endothelial cells. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines, such as IL-1α,

IL-1β, TNFα and IFN, are all shown to induce PS exposure without causing cytotoxicity. Hydrogen peroxide is also shown to be a strong inducer of PS, and inflammatory cytokines and hypoxia-reoxygenation are shown to have greater than additive effects, supporting the inventors' surprising findings that factors in tumors interact to give amplified effects on PS-exposure on the normal tumor vascular endothelial cells *in vivo*. All such information, and its effective use to provide the kits of the claimed invention, exists in the present application but not in the cited art.

The rejection under 35 U.S.C. § 103(a) is thus overcome and should be withdrawn.

VI. Double Patenting Rejection Over the '862 Application

The Fourth Action maintains the provisional rejection of all examined claims under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over "pending claim" of the co-pending Application Serial No. 09/351,862 ("the '862 application"; Attorney Docket No. 4001002282). In regard to the present claims and the claims in the '862 application, the Action states:

> "Although the conflicting claims are not identical, they are not patentably distinct from each other because they are both directed to kits comprising antibodies directed to aminophospholipids and a second anti cancer agent"
>
> Fourth Action at page 5; emphasis added.

Applicants earlier pointed out that the present claims are not directed to kits comprising *antibodies* directed to aminophospholipids, but to kits comprising a targeting agent-therapeutic agent constructs, *i.e.*, to anti-aminophospholipid *immunoconjugates*[5]. The Action appears to appreciate the scientific distinction, but maintains that "the scope of the instant claims overlap with the scope of the pending application '862" (Fourth Action at pages 5 and 6).

[5]The Action is in error in the statement that claim 1 in the present application contains "a first antibody ..." (Fourth Action at page 6). Claim 1 in fact recites "a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent".

A provisional double patenting between two co-pending applications, by its very nature, requires the Office to consider the claims in the two applications to be legally obvious, each over the other. MPEP 804 at page 800-19. This is evident in the present rejection, which refers to the conflicting claims not being patentably distinct "from each other" and states that the claims "overlap" (Fourth Action at pages 5 and 6).

However, there is no provisional double patenting rejection over the present application in the '862 application. In the absence of a provisional double patenting rejection in the '862 application, the present rejection is *prima facie* improper and must be withdrawn as legally and procedurally inconsistent. MPEP 804. To maintain the present rejection, the Office must enter a provisional double patenting rejection in the '862 application (which would have to be made as part of a Non-Final Office Action, being a new ground of rejection not necessitated by Applicants' amendment or untimely submission of references).

VII. Conclusion

This is a complete response to the referenced Official Action. In conclusion, Applicants submit that, in light of the foregoing remarks, the present case is in condition for allowance and such favorable action is respectfully requested. Should Examiner Sharareh have any questions or comments, or identify any informalities, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,

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Phosphatidylserine is a marker of tumor vasculature and a potential target for anti-cancer drugs[1]

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Running title: PS is a marker of tumor vessels

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Abbreviations used in this paper are: TF, tissue factor; VCAM-1, vascular cell adhesion molecule 1; IL-1a, interleukin-1 alpha; DPBS, Dulbecco's phosphate buffered saline; HRP; horseradish peroxidase; AP, alkaline phosphatase; SMC, smooth muscle cells; SCID, severe combined immunodeficient; PS, Phosphatidylserine; CL, cardiolipin; ROS, reactive oxygen species.

ABSTRACT

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides on the inner leaflet of the plasma membrane under normal conditions. PS translocation to the external side of the membrane (PS exposure) is associated with apoptosis, necrosis, cell injury, cell activation and malignant transformation. We previously reported that endothelial cells in tumor vessels of Hodgkin's lymphoma implanted in mice express PS on their external surface. Endothelial cells in normal tissues did not express detectable amounts of PS. In the present study, we analyzed five additional tumor models for the exposure of PS on tumor vasculature and investigated a potential mechanism underlying this phenomenon. Anti-PS antibody specifically localized to tumor blood vessels in all tumors (HT29 human colon adenocarcinoma, NCI-H358 human lung carcinoma, B16 mouse melanoma, 3LL mouse lung carcinoma and Colo 26 mouse colon carcinoma). No localization was detected on normal endothelium. An isotype-matched control antibody directed against a different negatively charged lipid, cardiolipin, did not localize to either tumor or normal endothelium. Frozen tumor sections were examined for the presence of apoptotic cells, using a double labeling technique that detected a pan-endothelial cell marker and the apoptosis markers, active caspase 3 and fragmented DNA (Tunel assay). Neither apoptosis marker was present in tumor endothelium, indicating that PS-positive tumor vessels are seldom apoptotic. Externalization of PS also did not correlate with the maturation status of the vessels, as an abnormal pericytic network was equally evident around PS-positive and PS-negative tumor vessels. Various factors and tumor-associated conditions known to be present in the tumor microenvironment were examined for their ability to cause PS translocation in cultured endothelial cells. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines all induced PS exposure without causing cytotoxicity. Hydrogen peroxide was also a strong inducer. Possibly, conditions inside tumors generate reactive

oxygen species that induce PS exposure. Combined treatment with inflammatory cytokines and hypoxia/reoxygenation had greater than additive effects, suggesting that factors may interact to give amplified effects on PS-exposure on tumor endothelium *in vivo*. Since PS is absent from the outer surface of normal endothelium, its exposure on tumor vessels could potentially be utilized for tumor vessel targeting and imaging.

INTRODUCTION

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides in the inner leaflet of the plasma membrane under normal conditions (1; 2). PS asymmetry is maintained by an ATP-dependent aminophospholipid translocase that is responsible for inward movement of aminophospholipids (3-5). Loss of PS asymmetry results from the outward movement of PS in the plasma membrane and is caused either by inhibition of the translocase (6; 7) or activation of scramblase, a Ca^{2+} dependent enzyme that transports lipids bidirectionally (8; 9). Loss of PS asymmetry is observed under different pathological and physiological conditions, including programmed cell death (10; 11), cell aging (12), intercellular fusion of myoblasts (13) and trophoblasts (14), cell migration (15; 16), activation of platelets (17-19) and cell degranulation (20). Endothelial cells externalize PS in response to thrombin (21) hyperlipidemia (22), viral infection (23), non-lytic concentrations of complement proteins C5b-9 (24) or exposure to calcium ionophore A23187 and PMA (25). Spontaneous PS exposure has been also observed in malignant cells in the absence of exogenous activators or cell injury (26-28).

Several major consequences follow membrane PS exposure. Phagocytic macrophages recognize, attach and eliminate PS-positive senescent and apoptotic cells (29; 30). PS also mediates attachment of T lymphocytes to thrombin-activated endothelial cells (21). The complement system is activated by PS and contributes to the lysis of PS-positive cells (31). Finally, PS exposure contributes to a procoagulant shift on the endothelium (1; 11) by providing a negatively charged lipid surface for assembly and activation of coagulation complexes (32; 33).

A mouse monoclonal IgM, 3SB, directed against PS has been raised and characterized by Rote and colleagues (17). The antibody binds to PS but not to phosphatidylcholine, phosphatidylethanolamine or cardiolipin (CL). 3SB binds to PS-coated ELISA plates in the presence or absence of serum, indicating that PS binding does not require cofactors. 3SB binds to cells having exposed PS (17). It is possible that it recognizes hexagonally packed PS, which has been reported to be antigenic (34).

We previously discovered that 3SB localized to tumor blood vessels in mice bearing human Hodgkin's disease tumors. This finding indicated that endothelial cells in Hodgkin's tumors, in contrast to those in normal tissues, expressed PS on the external surface of their plasma membrane. In the present study, we determined whether vascular PS exposure is observed in other types of tumors, and investigated the causes of PS translocation. PS exposure on tumor vasculature was present in all of six different tumors growing in mice. PS exposure was not due to apoptosis of tumor endothelium, or to irregularities in their coating with perivascular cells. Studies with cultured endothelial cells showed that hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines caused PS exposure without causing cytotoxicity. Hydrogen peroxide did likewise. Hypoxia/reoxygenation, acidity, thrombin and inflammatory cytokines may therefore act individually or collectively in tumors to generate peroxide ions and other reactive oxygen species that induce PS exposure on tumor endothelium.

PS on tumor vessels may provide a target molecule for the vascular targeting or imaging of vessels in solid tumors. Annexin V, an endogenous PS-binding ligand, has been used successfully to image PS-expressing activated platelets in thrombi (35), apoptotic cells in rejecting cardiac allografts, cyclophosphamide-treated lymphomas and anti-Fas antibody-

treated livers in rodents (10). Anti-PS antibodies may be directly cytotoxic to tumor vasculature, or mediate the binding of cytotoxic or coagulation factor to tumor vessels complement, host cells. Also, anti-PS antibodies, annexin V and other ligands that bind specifically to PS on tumor endothelial cells might be used to deliver a cytotoxic drug, radionuclide or coagulant to tumor vessels. Vascular targeting agents directed against markers on mature blood-transporting vessels in tumors have caused destruction of tumor vasculature and major tumor regressions in other systems (36-38). The present studies suggest the use of PS-directed antibodies and immunoconjugates for the vascular targeting or imaging of tumor vessels in man.

MATERIALS AND METHODS

Materials. $Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's tissue culture medium and Dulbecco PBS containing Ca^{2+} and Mg^{2+} were obtained from Gibco (Grand Usland, NY). Fetal calf serum was obtained from Hyclone (Logan, Utah). O-phenylenediamine, hydrogen peroxide and thrombin were from Sigma (St. Louis, MO). Flat bottom plates with 24 wells were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ). Recombinant murine interleukin-1 alpha, beta and tumor necrosis factor alpha (TNF α) were purchased from R&D Systems (Minneapolis, MN). Interferon of Universal Type I (hybrid protein that substitutes for all types of interferons) was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Recombinant hepatocyte growth factor (HGF or scatter factor) and actinomycin D were from Calbiochem. Recombinant VEGF, PDGF-BB, $TGF\beta_1$, interleukin–6 (IL-6),

interleukin-8 (IL-8), interleukin-10 (IL-10) and FGF-1 were purchased from PeproTech (Rocky Hill, NJ).

Antibodies

Mouse monoclonal anti-PS and anti-CL IgM antibodies were raised as described (17). Both antibodies have been extensively characterized with regard to the specificity of their binding to PS and CL respectively (17). MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. E. Butcher (Stanford University, CA) and served as a positive control for immunohistochemical studies. Details of this antibody have been published (39). Rabbit anti-rat immunoglobulin, rat-anti mouse immunoglobulin and goat-anti mouse immunoglobulin secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) were purchased either from Daco (Carpinteria, CA) or from Jackson Immunoresearch Labs (West Grove, PA).

Cells

L540Cy Hodgkin lymphoma cells, derived from a patient with end-stage disease, were provided by Prof. V. Diehl (Köln, Germany). HT29 human colon adenocarcinoma and NCI-H358 human non-small cell lung carcinoma were provided by Dr. Adi Gazdar (Southwestern Medical Center, Dallas, TX). B16 mouse melanoma and 3LL mouse lung carcinoma were obtained from American Type Cell Collection (Rockville, MD). Colo 26 mouse colorectal carcinoma was a gift from Dr. Ian Hart (ICRF, London, UK). The mouse brain endothelioma, bEnd.3, was provided by Prof. Werner Risau (Max Plank Institution, Munich, Germany).

METHODS

Tissue Culture

bEnd.3 cells, adult bovine aortic endothelial (ABAE) cells and all tumor cells except L540Cy lymphoma cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 units/ml penicillin G and 2 ug/ml streptomycin. L540Cy cells were maintained in RPMI 1640 containing the same additives. Cells were sub-cultured once a week. Trypsinization of bEnd.3 cells was performed using 0.125% trypsin in PBS containing 0.2% EDTA. For *in vitro* studies, endothelial cells were seeded at a density of $1x10^4$ cells/ml in 1 ml of culture medium in 24 well plates and incubated 48-96 hours before being used in the assays. Medium was refreshed 24 hours before each experiment.

Growth of tumor cells *in vivo*

For localization studies, $2x10^7$ L540 human Hodgkin's lymphoma cells or $1x10^7$ cells of other tumor types were injected subcutaneously into the right flank of SCID mice (Charles River, Wilmington, MA). Tumors were allowed to reach a volume of 0.4-0.7 cm^3. A minimum of three animals per tumor group was used. Experiments were performed at least three times.

Detection of PS exposure on tumor endothelium *in vivo*

Detection of exposed PS *in vivo* was performed essentially as previously described (37). Briefly, anti-PS or anti-CL mouse IgM antibodies (30 ug/mouse) were injected intravenously in 200 ul of saline. Thirty minutes later mice were sacrificed and their blood circulation was exsanguinated and perfused with heparinized saline as previously described (40). All major organs and tumor were harvested and snap-frozen for preparation of

cryosections. Mouse IgM was detected using goat anti mouse IgM (μ specific) - HRP conjugate followed by development with carbazole (41). The number of positive vessels per high power field was determined at magnification of x 40. At least 10 random fields per section were examined and the average percentage of positive vessels was calculated.

Detection of apoptosis in tumors *in situ*

The blood circulation of mice was perfused with heparinized saline as previously described (40). Tumors were dissected out and snap-frozen for cryosectioning. Cytosolic and nuclear alterations characteristic for apoptotic cells were detected immunohistochemically by using two markers: active caspase-3 (42) and fragmented DNA (43). Active caspase-3 was detected by a rabbit anti-caspase-3 specific antibody (R&D, Minneapolis, MN) followed by incubation with anti-rabbit IgG conjugated to alkaline phosphatase (AP, Pierce, Rockford, IL). Other tumor sections were analyzed by Tunel assay (ApopTag kit, Oncor, MD) using anti-digoxigenin–alkaline phosphatase conjugate as a detecting reagent. To determine whether tumor endothelial cells express apoptotic markers, sections were sequentially labeled with MECA 32 (anti-endothelial marker) and anti rat-HRP secondary antibody, followed by either anti-caspase-3 antibody or Tunel assay and AP-conjugated secondary reagents. Vessels were visualized by their brown color, using Stable DAB (Research Genetics, Huntsville, AL) as a substrate. Apoptotic cells were identified by their pink-purple color (kit from Research Genetics) created by the phosphatase activity of secondary reagents detecting markers of apoptosis. The pink-purple stain was clearly distinguishable from the brown stain, if both markers coincided. These conditions of double labeling permitted the sequential detection of both enzymes.

Detection of pericytes on tumor frozen sections

To characterize interactions between PS-positive vessels and pericytes, tumor sections were double-labeled by goat anti mouse IgM-HRP (to identify anti-PS localized antibody) followed by anti α-smooth muscle cell actin (α-SMC) antibody (Daco, Carpinteria, CA), a marker of pericytes and vascular smooth muscle cells (SMC)(44; 45). The peroxidase activity was detected by Stable DAB and resulted in brown color. The pericytic marker was detected by anti mouse IgG-AP conjugate and resulted in a pink-purple color. Substrates for both enzymes were from Research Genetics. In other experiments, the endothelium was first detected by MECA 32 IgG and visualized by DAB, followed by anti α-SMC antibody and AP-mediated detection of pericytes/SMC.

Iodination of annexin V

Recombinant human annexin V was purified from E. coli transformed with ET12a-PAP1 plasmid (a gift from Dr. J. Tait, University of Washington, Seattle). The purity of the protein and the binding to PS were confirmed on SDS-PAGE and on PS-coated plastic, respectively. Rabbit polyclonal, affinity-purified anti-annexin V antibody was used to detect annexin V bound to PS. Annexin V was radiolabeled with ^{125}I using Chloramine T as described by Bocci (46). The specific activity was approximately $1x10^6$ cpm per ug of protein, as measured by a Bradford assay (47).

Effect of growth factors, cytokines, inflammatory mediators, hydrogen peroxide, hypoxia and acidic pH on translocation of PS in cultured endothelial cells

Endothelial cells were treated with cytokines or growth factors at the concentrations listed in Table 3. All reagents were diluted in medium containing 10% serum and incubated with the cells at 37°C for 24 hours. To study the effect of hypoxia, cells were seeded on 24 well

plates and were incubated in a humidified normoxic atmosphere (21% O_2, 5% CO_2) for 48 hours before being transferred to a humidified hypoxic atmosphere (1% O_2, 5% CO_2, 94% N_2) in a sealed chamber (Billups Rothenberg inc, Del Mar, Ca). Cells were incubated in a hypoxic chamber for 24 hours at 37°C and the cells were compared to a parallel culture maintained under normoxic conditions. In some experiments, IL-1α (10 ng/ml) and TNFα (20 ng/ml) were added to medium prior to transfer to a hypoxic chamber.

To examine the effect of an acidic microenvironment, cells were exposed to the growth medium lacking bicarbonate, which was adjusted to different pHs (ranged between 7.3 to 6.2) with the required amount of HCl. Cells were incubated at 37°C in the absence of CO_2. Preservation of the medium pH in the presence of cell monolayer during 24 hours period was confirmed in each experiment. Under these experimental conditions all tested reagents were non-toxic to either bovine or mouse endothelial cells and had no effect on cell morphology or viability of the attached monolayer.

Detection of exposed PS on cultured endothelial cells by ^{125}I-labeled annexin V

After treatment with the reagents described above, treated and control cells were incubated with 7.1 pmoles of ^{125}I-labeled annexin V (200 ul/well) in the binding buffer. After 2 hours incubation at room temperature, cells were washed extensively and dissolved in 0.5 M of NaOH. The entire volume of 0.5 ml was transferred to plastic tubes and counted in a gamma counter. Non-specific binding was determined in the presence of 5 mM EDTA and was subtracted from experimental values. The results were expressed as net pmoles of cell-bound annexin V, normalized per $1x10^6$ cells. Maximal binding of annexin V was determined on cells simultaneously treated with actinomycin D and TNFα (50 ng/ml of each component). As has been previously reported, combination of the above agents causes apoptosis and PS exposure in the 100 percent of the treated endothelial cells (48).

Basal binding of ^{125}I-annexin V to untreated cells was determined in the presence of medium with 10% serum. The amount of ^{125}I-annexin V that bound to the untreated cultures was subtracted from that in the treated cultures. The specific increase in the amount of externalized PS was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Each experiment was performed in duplicate and was performed at least three times.

Detection of exposed PS on endothelial cells *in vitro* by biotinylated annexin V

Endothelial monolayers were washed with DPBS containing Ca^{2+} and Mg^{2+} and fixed with 0.25% of glutaraldehyde diluted in the same buffer. Excess aldehyde groups was quenched by incubation with 50 mM of Na_4Cl for 5 minutes. Cells were washed with DPBS (containing Ca^{2+}, Mg^{2+} and 0.2% gelatin) and incubated with 1 ug/ml of biotinylated annexin V (Pharmingen, San Diego, CA). After 2 hours of incubation, cells were washed with 0.2% gelatin buffer incubated with streptavidin-HRP (1:500 dilution). Detection of PS *in situ* was confirmed by staining with anti-PS IgM. Anti-CL IgM and streptavidin were used as negative controls in these assays. All steps were performed at room temperature. PS-positive cells were detected by addition of carbazole substrate, resulting in insoluble red-brownish precipitate. The number of positive cells per high power field was determined and expressed as a percent of the total number of cells. Six random fields were scored per well and the average was calculated.

RESULTS

PS is a marker of tumor vasculature.

PS exposure on vascular endothelium *in vivo* was detected by a specific anti-PS mouse IgM, (3SB) as previously described (37). A class and species matched antibody, D11 (17),

directed against a different negatively charged phospholipid, CL, served as a negative control. CL exclusively resides in the membrane of mitochondria (49). This anti-CL monoclonal antibody recognizes CL, not PS or other lipids (17).

All six tumors included in this study contained PS-positive vessels (Fig.1 and Table 1). The percentage of PS-positive vessels ranged from 40% in B16 tumors to 10% in Colo 26 tumors. Anti-PS IgM was present on the luminal surface of capillaries and venules in all regions of the tumors. PS-positive vessels appeared to be particularly prevalent in and around regions of necrosis. Positive vessels usually did not show morphological abnormalities that were apparent by light microscopy. Occasional vessels located in necrotic areas showed morphological signs of deterioration. Detection of PS by 3SB was specific since no staining of tumor endothelium was observed with the anti-CL antibody. Anti-PS antibody (but not anti-CL antibody) also localized to necrotic and apoptotic tumor cells. No vascular localization of anti-PS or anti-CL antibodies was observed in normal organs other than the kidneys (Table 1). In the kidneys, tubules were stained in both anti-PS and anti-CL recipients, presumably because of secretion of IgM through this organ. These findings demonstrate that PS is present on the luminal surface of vascular endothelial in various tumors but not in normal tissues.

PS-positive tumor vessels are not apoptotic

A double labeling technique was used to identify apoptotic endothelial cells in tumor sections. Apoptotic cells were identified with two independent markers: an active form of caspase-3, which identifies cytosolic changes in dying cells (42), and fragmented DNA, which identifies cells having nuclear alterations (43). Active caspase-3 was detected by a specific antibody. Fragmented DNA was visualized by Tunel assay (43). Cells positive for apoptotic markers were stained pink by AP-labeled secondary reagents. Endothelial cells in blood vessels were stained brown by HRP-labeled by a pan-endothelial antibody, MECA

32. Both colors were clearly visible on the same cells, if endothelial and apoptotic markers coincided.

Endothelial cells in five out of six types of tumors (HT29, H358, B16, Colo 26, L540) did not display either of the apoptosis markers (Fig. 2, Table 2). The sixth type of tumor, 3LL, displayed a few apoptotic endothelial cells that were located in necrotic areas. In contrast, apoptotic malignant cells were common in all types of tumors. The percentage of apoptotic tumor cells ranged from 1-2% in L540 tumors to 12.6-19.6% in 3LL tumors. There was broad correspondence between the number and location of tumor cells that stained positively for active caspase 3 and for fragmented DNA. However, cells displaying active caspase 3 were about 1.5 times as abundant as those with fragmented DNA, probably because active caspase 3 is an earlier and less specific marker of apoptosis than is fragmented DNA.

Lack of correlation between PS-positive tumor vessels and abnormal distribution of pericytes

We explored whether abnormalities in the perivascular cell coating of tumor vessels might account for their PS exposure by determining whether there was a correlation between the integrity of the coating by pericytes and the positivity for externalized PS. Frozen tumor sections from mice that had been injected with anti-PS antibody or anti-CL antibody were double labeled for the presence of the vascular endothelial cell marker, MECA 32, and a pericytic cell/SMC marker, αSMC-actin. Endothelial cells were stained brown while pericytic cells were stained pink. PS-positive tumor vessels were identified in sequential sections by staining for localized anti-PS IgM (brown) followed by identification of pericytes (pink). In contrast to the continuous pericytic layer surrounding vascular

endothelial cells in normal tissues, vessels in all tumors had a discontinuous and disorganized network of pericytic cells (Fig. 3). Vessels in B16 melanomas and Colo 26 carcinomas almost completely lacked pericytes. HT29 and H358 carcinomas contained pericytes/SMC that were often totally separated from endothelial cells. The L540 lymphoma was the only tumor of those examined that had a major percentage (about 40%) of vessels in which pericytic cells were properly attached.

There was no correlation between irregularities in pericytic cells and exposure of PS on tumor vessels (Fig. 3). PS-positive and PS-negative tumor endothelium had equal proportions of coated vessels, uncoated vessels and vessels with partly detached pericytic layers. Abnormalities in pericyte coating, therefore, do not appear to be the cause of PS translocation on tumor endothelium.

Inducers of PS translocation by endothelial cells *in vitro*

Endothelial cells *in vitro* were treated with non-toxic concentrations of various factors and conditions that are present in the microenvironment of many tumors (50-54). The factors included: angiogenic factors (VEGF, HGF and bFGF) tumor and host cell–derived inflammatory and pro-angiogenic cytokines (1L-1α, IL-1β, TNFα, IL-6, IL-8, IL-10 and interferons), thrombin, hypoxia/reoxygenation , oxygen-reactive species and low pH. These conditions are all known to perturb endothelial cells and could be considered as potential cause of PS translocation. Activation, perturbation and injury of endothelium by these factors and conditions are associated with a rise of intracellular Ca^{2+} concentration. The rise in intracellular Ca^{2+} might activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6), leading to accumulation of PS on the external side of the membrane.

Mouse bEnd.3 or bovine ABAE cells were treated for 24 hours with different concentrations of potential inducers of PS translocation. PS externalization was quantified by measuring ^{125}I-annexin V binding. The amount of annexin V binding was compared with that of cells in which apoptosis of 100% of cells had been induced by combined treatment with actinomycin D and TNF-α. Actinomycin D and TNF-α induced the binding of 6.2 pmoles of annexin V per 10^6 cells (3.8×10^6 molecules of annexin V per cell) on both cell types, in good agreement with literature reports (55). This value was taken as the maximal amount of externalized PS.

Untreated cells were largely devoid of externalized PS, as judged by annexin V or anti-PS antibody binding (Table 3, and Fig. 4A). The basal binding in the presence of growth medium alone was 0.44 and 0.68 pmoles of ^{125}I-annexin V for ABAE and bEnd.3 cells, respectively. This corresponds to 7.06% and 10.9% of the maximal binding for ABAE and bEnd.3 cells, respectively, which correlated well with the finding that approximately 10% of cells bound biotinylated annexin V under the same conditions. VEGF, HGF, FGF, TGFβ$_1$, PDGF, IL-6, IL-8 and IL-10 did not increase binding of ^{125}I-annexin V above the basal level for untreated cells. Inflammatory mediators (IL-1α, IL-1β, TNFα and interferon) caused a small but reproducible increase in PS translocation that ranged from 6 to 8% of the maximal level for ABAE cells and from 7 to 14% for bEnd3 cells (Table 3). Hypoxia/reoxygenation, thrombin or acidic external conditions (pH 6.8-6.6) induced a moderately high externalization of PS that ranged from 8 to 20% of the maximal level for ABAE cells and from 17 to 22% of the maximal level for bend.3 cells. The largest increase in PS translocation was observed after treatment with 100 to 200 uM of hydrogen peroxide. This treatment caused nearly complete (95%) externalization of PS in both cell types as judged by ^{125} I-annexin V binding (Table 3). More than 70% of ABAE and bEnd.3

cells bound biotinylated annexin V, as judged immunohistochemically (Fig. 4B). PS-expressing endothelial cells generated by treatment with hypoxia/reoxygenation, thrombin, acidity, TNFα, IL-1 or H_2O_2 remained attached to the matrix during time period of the assay (24 hours), retained cell-cell contact (Fig. 4B) and retained their ability to exclude trypan blue dye. Normal PS orientation was restored 24 to 48 hours later in the majority of the cells. These results indicate that mild oxidative stress, created by direct application of H_2O_2 or indirectly by hypoxia/reoxygenation, acidity, thrombin, or inflammatory cytokines, triggers a transient translocation of PS on viable endothelial cells.

Combined Effects of Inflammatory Cytokines and Hypoxia/Reoxygenation on PS-Exposure by Endothelial Cells *In Vitro*

Enhanced PS exposure was observed when ABAE cells were subjected to hypoxia/reoxygenation in the presence of IL-1α or TNFα. In the absence of the cytokines, hypoxia/reoxygenation increased PS-exposure by ABAE cells to 15% of the maximum level for cells treated with apoptotic concentrations of actinomycin D and TNFα. In the presence of subtoxic concentrations of IL-1α or TNFα, hypoxia/reoxygenation increased PS-exposure to 26% and 33% respectively of the maximum (Fig. 5). Cytokines in the absence of hypoxia/reoxygenation increased PS-exposure by less than 7% indicating that the combination of cytokines and hypoxia/reoxygenation had greater than additive effects on PS-exposure. Thus, in tumors, the PS-inducing effect of hypoxia/reoxygenation may be amplified by inflammatory cytokines and possibly by such other stimuli as acidity and thrombin.

DISCUSSION

The major finding to emerge from this study is that vascular endothelial cells in tumors externalize PS to their luminal surface where it can be bound by a specific anti-PS antibody *in vivo*. PS is absent from the external surface of vascular endothelial cells in normal tissues, suggesting that PS-recognizing antibodies, annexin V and other ligands might be used for delivering cytotoxic drugs, coagulants or radionuclides for the selective destruction or imaging of vessels in solid tumors.

PS-positive tumor endothelium appears to be viable. It does not display markers of apoptosis, it is morphologically intact and metabolically active, as indicated by its expression of VCAM-1, E-selectin and other rapidly turned-over proteins. Although often regarded as an indicator of apoptosis, PS exposure has been observed in several types of viable cells, including malignant cells (27; 28; 55), activated platelets (17), and embryonic trophoblasts at various stages of migration, matrix invasion and fusion (14). Recent studies suggest that constitutive PS exposure on malignant cells may occur because expression of truncated (less active) aminophospholipid translocase, as detected by epitope-specific antibodies against the putative PS translocase, ATPaseII[4]. Lack of correlation between PS exposure and commitment to cell death has been also shown on pre-apoptotic B lymphoma cells that restored PS asymmetry and grew normally after removal of pro-apoptotic stimulus (56). In normal viable cells, PS exposure is probably triggered by surface events, such as ligand-receptor interactions, that induce Ca^{2+} fluxes into the cells (57). Ca^{2+} fluxes activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6).

To shed light on the mechanism of PS exposure on tumor endothelial cells, a series of experiments was performed in which endothelial cells *in vitro* were treated with various factors and conditions known to be present in the tumor microenvironment. The objective was to identify factors that induce PS exposure on endothelial cells without causing cytotoxicity in order to mimic the situation in tumors *in vivo*. Hypoxia followed by reoxygenation, acidity, and thrombin increased PS exposure on viable endothelial cells to between 10 and 22% of the level seen when all cells are apoptotic. Inflammatory cytokines (TNFα and IL-1α) also caused a weak but definite induction of PS exposure. The possibility that these conditions are in fact the major inducing stimuli in tumors *in vivo* is suggested by the following: i) PS positive endothelium is prevalent in and around regions of necrosis where hypoxia, acidity, thrombosed blood vessels, and infiltrating host leukocytes are commonly observed; ii) the finding that hypoxia/reoxygenation amplifies the weak PS-exposing activity of TNFα and IL-1 on endothelial cells *in vitro* (Fig. 5) correlates with the situation *in vivo* in tumors where hypoxia and cytokine-secreting tumor and host cells co-exist; iii) hypoxia/reoxygenation and thrombin have been reported to generate reactive oxygen species (e.g. peroxides) in endothelial cells through activation of NADPH oxidase-like membrane enzyme (58; 59). Hydrogen peroxide was the most powerful inducer of PS exposure on cultured endothelial cells found in the present study, providing indirect support for the involvement of reactive oxygen species. Based on these findings we propose the following cascade of events leading to PS exposure on tumor vessels, which indirectly contributes to tumor-associated thrombosis (Fig. 6). Hypoxia/reoxygenation in combination with inflammatory cytokines, thrombin and acidity are responsible in part for generation of ROS by endothelial and tumor cells. ROS are also produced by infiltrating host cells (macrophages, neutrophils and granulocytes) that are

attracted by necrosis and tumor-derived cytokines. Tumor endothelial cells respond to rising ROS concentration by transferring PS to the external side of the membrane. The externalized PS provides the negative phospholipid surface upon which coagulation factors concentrate and assemble. This confers the procoagulant status to the tumor endothelium that has long been recognized. PS also provides an attachment site for circulating macrophages (29), T lymphocytes (21) and polymorphonuclear cells that assist in leukocyte infiltration into tumors. Adherence of activated macrophages, polymorphonuclear cells and platelets to PS on tumor endothelium may lead to further secretion of reactive oxygen species and further amplification of PS exposure.

Anti-PS antibodies might be used for cancer therapy in several ways. Unconjugated antibodies might directly suppress tumor endothelial cell growth or survival by interfering with critical PS-dependent surface functions. This hypothesis is supported by the studies on viable B cells demonstrating that PS neutralization inhibits signaling from the cell surface via B cell receptor (57). Anti-PS antibodies might also mediate toxicity by binding complement and cytotoxic cells, by inducing apoptosis or by promoting pro-thrombotic status of tumor vascular endothelium. In certain autoimmune disorders, anti-aminophospholipid antibodies cause normal tissue damage by analogous mechanisms (60-62). Also, anti-PS antibodies or ligands might be linked to various effector molecules (e.g. cytotoxic drugs, radionuclides, coagulants) to create vascular targeting agents that destroy or occlude blood vessels in solid tumors. Such agents have been shown to be highly effective, and sometimes curative, in mice with large solid tumors (36; 38).

PS on tumor vessels is attractive as a target for several reasons: it is abundant (minimum of 3×10^6 molecules per cell); it is on the luminal surface of tumor endothelium, which is

directly accessible for binding by vascular targeting agents in the blood; it is present on a high percentage of tumor endothelial cells in diverse solid tumors, and it is absent from endothelium in all normal tissues examined to date. Unconjugated antibodies, vascular targeting agents and imaging agents directed against PS on tumor vasculature potentially could have utility for cancer treatment in man.

Acknowledgment

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Table 1 Specific localization of anti-PS and anti-CL antibodies to tumor vessels mice

Tissue	Anti-PS[a]	Anti-cardiolipin
L540 tumor	++[b]	-
H358 tumor	++	-
HT29 tumor	+	-
B16 tumor	+++	-
3LL tumor	++	-
Colo 26 tumor	++	-
Adrenal	-	-
Brain	-	-
Heart	-	-
Kidney	-[c]	-[c]
Intestine	-	-
Liver	-	-
Lung	-	-
Pancreas	-	-
Spleen	-	-
Testis	-	-

[a] Localization of anti-PS or anti-CL antibody in tumor bearing mice was determined by injecting the antibody, perfusing the blood circulation of the mice with saline and detecting the antibody on sections of the tissues by using an anti-mouse IgM - peroxidase conjugate.

[b] Intensity of staining was compared to pan-endothelial marker MECA 32; - indicates no staining; + weak (fewer than 5% positive vessels) ; ++ moderate (5 to 20 %); +++ (20 to 40 %) strong.

[c] Non-antigen specific tubular staining was visible in both anti-PS and anti-CL recipients.

Table 2 **Expression of apoptotic markers in tumors**

Tumor type	Active caspase-3		Tunel assay	
	Tumor cells (% of total) [a]	Tumor vessels	Tumor cells (% of total)	Tumor vessels
3LL	19.8	<1.0 [b]	12.6	0
HT29	13.7	0	7.8	0
H358	5.8	0	4.3	0
Colo 26	5.3	0	4.1	0
B16	4.2	0	3.5	0
L540	2.3	0	1.6	0

[a] The percentage of tumor cells or tumor blood vessels that were positive for either caspase-3 or Tunel was determined in ten high power fields per section. The fields were randomly selected along two perpendicular directions from the edges through the center of the tumor. The average of the percentage of positive cells or vessels in tumor from 3 mice is presented.

[b] Occasional vessels (1 of >100) in the necrotic area of 3LL tumor displayed both markers of apoptosis.

Table 3 Effect of cytokines, growth factors and stress conditions on exposure of PS on endothelial cells *in vitro*.

Treatment	Concentration[a]	^{125}I-Annexin V (% of Max binding)[b]	
		ABAE cells	bEnd.3 cells
Medium with 10% serum	N/A[c]	0	0
Actinomycin D + TNF α	50 ng/ml each	100	100
VEGF	20 ng/ml	0	0
βFGF	20 ng/ml	0	0
Scatter factor	40 ng/ml	0	0
TGF β_1	20 ng/ml	0	0
PDGF-BB	20 ng/ml	0	0
IL-10	20 ng/ml	0	0
IL-8	20 ng/ml	0	0
IL-6	20 ng/ml	0	0
IL-1α	10 ng/ml	6.4	7.5
IL-1β	10 ng/ml	5.8	5.5
Interferon	40 ng/ml	8.6	2.8
TNFα	20 ng/ml	7.4	13.7
Thrombin	50 nM	8.8	17.4
Hypoxia	1% O_2	15.0	22.5
pH 6.6[d]	N/A	20.2	18.9
Hydrogen peroxide	100 uM[e]	95.5	98.4

[a] Concentrations of cytokines, growth factors and thrombin were selected from literature values to have maximal stimulatory effect on cultured endothelial cells. These concentrations did not cause toxicity over the period of the test (24 hours) as judged by morphological appearance, a lack of detachment, and a lack of uptake of trypan blue.

[b] Binding of ^{125}I-annexin V was performed as described under Methods. Basal binding was determined in the presence of growth medium alone. Maximal PS exposure was determined after induction of apoptosis by the combined treatment with actinomycin D and TNF α. Untreated ABAE and bEnd.3 cells bound 0.44 and 0.68 pmoles of ^{125}I-Annexin V, respectively. Maximal binding was 6.2 pmoles of ^{125}I-annexin V for both cell types (equivalent to 3.8×10^6 molecules per cell) The percentage of increase of annexin V binding was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Average of duplicates from three separate experiments is presented. SE was less than 5%.

[c] Not applicable.

[d] Cells were exposed to the growth medium lacking bicarbonate that had been adjusted to pH 6.6 with 1N HCl. Cells were incubated at 37^0C in the absence of CO_2.

[e] The maximal concentration of H_2O_2 that did not cause cytotoxicity under chosen conditions.

Figure Legends:

Fig. 1. Localization of anti-PS antibody to vascular endothelial cells in L540 human Hodgkin's lymphoma, 3LL murine lung carcinoma and B16 murine melanoma tumors in mice. Tumor-bearing SCID mice were injected intravenously with 20 ug of anti-PS or anti-CL mouse IgM. The blood circulation was perfused with saline one hour later. Mice were sacrificed one hour later and tumor and organs were harvested and snap-frozen. Mouse IgM was detected on frozen sections using goat anti-mouse IgM-peroxidase conjugate. Anti-PS antibody specifically localized to blood vessels (indicated by arrows) in all tumors. No localization was observed in mice injected with control anti-CL IgM.

Fig. 2. Lack of apoptotic vascular endothelial cells in 3LL and L540 tumors. Frozen sections of 3LL and L540 tumors were double labeled with a pan-endothelial cell antibody, MECA 32 to visualize blood vessels, and with anti-apoptotic markers detecting either active caspase-3 or fragmented DNA (Tunel assay). MECA 32 staining of vessels is indicated by the brown color (arrows). Expression of apoptotic markers is indicated by the pink color (arrowheads). Both apoptotic markers are present in some tumor cells but are absent from tumor endothelium.

Fig 3. Pericyte-endothelial cell interactions in PS-positive and PS-negative vessels in 3LL tumors. Mice bearing 3LL tumors were injected with 20 ug of anti-PS IgM. PS-positive vessels (*upper panel*) were detected as described above. PS-negative vessels (defined as MECA 32-positive, anti-PS-negative) were identified on serial sections of the same tumor (*lower panel*). Vessels were labeled brown with the pan-endothelial cell marker. Pericytes were identified with anti-αSMC actin antibody and were stained pink (arrowheads). Representative fields of PS-positive, pericyte-negative (*upper left*), PS-positive, pericyte-positive (*upper right*), PS-negative, pericyte-negative (*lower left*) and PS-negative, pericyte-positive (*lower right*) vessels are shown. Note the detachment of the pericytic layer from tumor endothelium and the uneven distribution of pericytes along tumor vessels (*upper and lower right*).

Fig. 4. Externalized PS on bEnd.3 mouse endothelial cells treated with 100 uM of hydrogen peroxide. A) untreated bEnd.3 cells; B) bEnd.3 cells after treatment with 100 uM of hydrogen peroxide. PS is absent from the surface of untreated cells but becomes externalized after treating the cells with H_2O_2. PS-positive cells remain attached to the substratum, retain cell-cell contact and other morphological signs of viability.

Fig. 5. Synergistic effect of hypoxia and inflammatory cytokines on PS exposure. bEnd.3 cells were treated for 24 hours with IL-1 α and TNF α under normoxic and hypoxic conditions. PS externalization was determined on viable endothelial monolayer by measuring binding of ^{125}I-annexin V. The increase in PS exposure was calculated as explained under "Materials and Methods".

Fig. 6. Hypothesis for induction of PS exposure on tumor vessels and its contribution to the procoagulant shift of the tumor endothelium.